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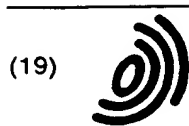
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(54) **L-glutamic acid-producing bacterium and method for producing L-glutamic acid**

(57) L-Glutamic acid is produced by culturing in a liquid culture medium a microorganism belonging to the genus *Enterobacter* or *Serratia* and having an ability to produce L-glutamic acid, which increases in an activity of enzyme catalyzing a reaction for L-glutamic acid bio-

synthesis, or which decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid, and collecting produced L-glutamic acid from the culture medium.

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Description

BACKGROUND OF THE INVENTION

[0001] The present invention relates to a novel L-glutamic acid-producing bacterium and a method for producing L-glutamic acid by fermentation using the same. L-Glutamic acid is an important amino acid as food, drugs and the like.

[0002] L-Glutamic acid has conventionally been produced by fermentation methods utilizing the so-called coryneform L-glutamic acid-producing bacteria which principally belong to the genera *Brevibacterium*, *Corynebacterium*, and *Microbacterium* or variants thereof ("Amino Acid Fermentation", Gakkai Shuppan Center, pp.195-215, 1986). As methods for producing L-glutamic acid by fermentation utilizing other bacterial strains, there have been known the methods utilizing microorganisms of the genera *Bacillus*, *Streptomyces*, *Penicillium* and the like (Unite States Patent No. 3,220,929); the methods utilizing microorganisms of the genera *Pseudomonas*, *Arthrobacter*, *Serratia*, *Candida* and the like (Unite States Patent No. 3,563,857); the methods utilizing microorganisms of the genera *Bacillus*, *Pseudomonas*, *Serratia* and the like or *Aerobacter aerogenes* (currently referred to as *Enterobacter aerogenes*) (Japanese Patent Publication (KOKOKU) No. 32-9393(1957)), the method utilizing variant strains of *Escherichia coli* (Japanese Patent Application Laid-Open (KOKAI) No. 5-244970(1993)) and the like.

[0003] Though the productivity of L-glutamic acid has considerably been improved by breeding of such microorganisms as mentioned above or improvements of production methods, it is still desired to develop a more inexpensive and more efficient method for producing L-glutamic acid in order to meet the expected markedly increasing future demand of the amino acid.

SUMMARY OF THE INVENTION

[0004] The object of the present invention is to find a novel L-glutamic acid-producing bacterium having a high ability to produce L-glutamic acid, thereby developing a more inexpensive and more efficient method for producing L-glutamic acid.

[0005] To achieve the aforementioned object, the present inventors intensively searched for and studied microorganisms having the ability to produce L-glutamic acid that are different from the previously reported microorganisms. As a result, they found that certain strains derived from microorganisms belonging to the genus *Enterobacter* or *Serratia* had a high ability to produce L-glutamic acid, and have completed the present invention.

[0006] Thus, the present invention provide:

(1) a microorganism belonging to the genus *Enterobacter* or *Serratia* and having an ability to produce L-glutamic acid and at least one of the following properties:

(a) the microorganism increases in an activity of an enzyme catalyzing a reaction for L-glutamic acid biosynthesis; and

(b) the microorganism decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid;

(2) a microorganism of the above (1) wherein the enzyme catalyzing the reaction for the L-glutamic acid biosynthesis is at least one selected from the group consisting of citrate synthase (abbreviated as "CS" hereinafter), phosphoenolpyruvate carboxylase (abbreviated as "PEPC" hereinafter), and glutamate dehydrogenase (abbreviated as "GDH" hereinafter);

(3) a microorganism of the above (2) wherein the enzyme catalyzing the reaction for the L-glutamic acid biosynthesis includes all of CS, PEPC and GDH;

(4) a microorganism of any one of the above (1) to (3) wherein the enzyme catalyzing the reaction branching from the pathway for L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid is α -ketoglutarate dehydrogenase (abbreviated as "aKGDH" hereinafter);

(5) a microorganism of any one of the above (1) to (4) which is *Enterobacter agglomerans* or *Serratia liquefacience*; and

(6) a method for producing L-glutamic acid which comprises culturing the microorganism as defined in any one of the above (1) to (5) in a liquid culture medium to produce and accumulate L-glutamic acid in the culture medium, and collecting the L-glutamic acid from the culture medium.

[0007] Because the microorganism of the present invention have a high ability to produce L-glutamic acid, it is considered to be possible to impart a further higher production ability to the microorganism by using the breeding techniques previously known for the coryneform L-glutamic acid-producing bacteria and the like, and it is expected to contribute

to developm nt of a more inexpensive and more efficient method for producing L-glutamic acid by appropriately selecting culture conditions and the like.

BRIEF EXPLANATION OF THE DRAWINGS

- [0008] Figure 1 shows construction of a plasmid pMWCPG having a *gltA* gene, a *ppc* gene and a *gdhA* gene.
 [0009] Figure 2 shows construction of a plasmid pSTVG having the *gdhA* gene.
 [0010] Figure 3 shows construction of a plasmid RSF-Tet having a replication origin of a wide-host-range plasmid RSF1010 and a tetracycline resistance gene.
 [0011] Figure 4 shows construction of a plasmid RSFCPG having the replication origin of the wide-host-range plasmid RSF1010, the tetracycline resistance gene, the *gltA* gene, the *ppc* gene and the *gdhA* gene.
 [0012] Figure 5 shows construction of a plasmid pMWCB having the *gltA* gene.
 [0013] Figure 6 shows a restriction map of a DNA fragment of pTWVEK101 derived from *Enterobacter agglomerans*.
 [0014] Figure 7 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sucA* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*. The upper sections: *Enterobacter agglomerans*, the lower sections: *Escherichia coli* (the same shall apply hereinafter).
 [0015] Figure 8 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sucB* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*.
 [0016] Figure 9 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sdhB* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*.
 [0017] Figure 10 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sucC* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*.

DETAILED DESCRIPTION OF THE INVENTION

- [0018] The present invention will be explained in detail hereinafter.
 [0019] The microorganism of the present invention is a microorganism belonging to the genus *Enterobacter* or *Serratia*, and having at least one of the following properties:
 (a) the microorganism increases in an activity of an enzyme catalyzing a reaction for L-glutamic acid biosynthesis; and
 (b) the microorganism decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid.
 [0020] Such a microorganism can be obtained by using a microorganism belonging to the genus *Enterobacter* or the genus *Serratia* as a parent strain, and imparting the properties of the above (a) and/or (b) to the microorganism. Examples of the microorganism belonging to the genus *Enterobacter* or the genus *Serratia* that can be used as the parent strain are listed below:

Enterobacter agglomerans
Enterobacter aerogenes
Enterobacter amnigenus
Enterobacter asburiae
Enterobacter cloacae
Enterobacter dissolvens
Enterobacter gergoviae
Enterobacter hormaechei
Enterobacter intermedius
Enterobacter nimipressuralis
Enterobacter sakazakii
Enterobacter taylorae
Serratia liquefacience
Serratia ontomophila
Serratia ficaria
Serratia fonticola
Serratia grimesii
Serratia proteamaculans
Serratia odorifera

Serratia plymuthica
Serratia rubidaea

[0021] More preferably, those bacterial strains listed below can be mentioned:

Enterobacter agglomerans ATCC 12287
Enterobacter agglomerans AJ13355
Serratia liquefacience ATCC 14460

[0022] The *Enterobacter agglomerans* AJ13355 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998, and received an accession number of FERM P-16644, and then transferred to an international deposition under the Budapest Treaty on January 11, 1999, and received an accession number of FERM BP-6614. The *Enterobacter agglomerans* ATCC 12287, and the *Serratia liquefacience* ATCC 14460 are available from ATCC.

[0023] The *Enterobacter agglomerans* AJ13355 is a strain isolated from soil in Iwata-shi, Shizuoka, Japan.

[0024] Physiological properties of AJ13355 are as follows:

- (1) Gram stain: Negative
- (2) Behavior for oxygen: Facultative anaerobe
- (3) Catalase: Negative
- (4) Oxidase: Positive
- (5) Nitrate reduction: Negative
- (6) Voges-Proskauer reaction: Positive
- (7) Methyl Red test: Negative
- (8) Urease: Negative
- (9) Indole production: Positive
- (10) Motility: Present
- (11) Hydrogen sulfide production in TSI culture medium: Slightly active
- (12) β -Galactosidase: Positive
- (13) Sugar assimilability:

Arabinose: Positive
 Sucrose: Positive
 Lactose: Positive
 Xylose: Positive
 Sorbitol: Positive
 Inositol: Positive
 Trehalose: Positive
 Maltose: Positive
 Melibiose: Positive
 Adonitol: Negative
 Raffinose: Positive
 Salicin: Negative
 Melibiose: Positive

- (14) Glycerose assimilability: Positive
- (15) Organic acid assimilability:

Citric acid: Positive
 Tartaric acid: Negative
 Gluconic acid: Positive
 Acetic acid: Positive
 Malonic acid: Negative

- (16) Arginine dehydratase: Negative
- (17) Ornithine decarboxylase: Negative
- (18) Lysine decarboxylase: Negative
- (19) Phenylalanine deaminase: Negative

(20) Pigment formation: Y llow

(21) Gelatin liquefaction: Positiv

(22) Growth pH: Not good growth at pH 4, good growth at pH 4.5 to 7

(23) Growth temperature: Good growth at 25°C, good growth at 30°C, good growth at 37°C, growth possible at 42°C, no growth at 45°C

[0025] From these bacteriological properties, AJ13355 is determined to be *Enterobacter agglomerans*.

[0026] In the working examples described hereinafter, *Enterobacter agglomerans* ATCC12287, *Enterobacter agglomerans* AJ13355, and *Serratia liquefacience* ATCC14460 were used as starting parent strains for obtaining strains which increase in the activity of the enzyme catalyzing the reactions for the L-glutamic acid biosynthesis, or strains which decrease in or are deficient in the activity of the enzyme catalyzing the reaction branching from the pathway for L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid. However, the sugar metabolism by any of bacteria belonging to the genera *Enterobacter* and *Serratia* is achieved via the Embden-Meyerhof pathway, and pyruvate produced in the pathway is oxidized in the tricarboxylic acid cycle under aerobic conditions. L-Glutamic acid is biosynthesized from α -ketoglutaric acid which is an intermediate of the tricarboxylic acid cycle by GDH or glutamine synthetase/glutamate synthase. Thus, these microorganisms share the same biosynthetic pathway for L-glutamic acid, and microorganism belonging to the genera *Enterobacter* and *Serratia* are encompassed within a single concept according to the present invention. Therefore, microorganisms belonging to the genera *Enterobacter* and *Serratia* other than species and strains specifically mentioned above also fall within the scope of the present invention.

[0027] The microorganism of the present invention is a microorganism belonging to the genus *Enterobacter* or the genus *Serratia* and having an ability to produce L-glutamic acid. The expression "having an ability to produce L-glutamic acid" as herein used means to have an ability to accumulate L-glutamic acid in a culture medium during cultivation. According to the present invention, the ability to produce L-glutamic acid is imparted by giving either one or both of the following characteristics:

(a) the microorganism increases in the activity of the enzyme catalyzing the reaction for the L-glutamic acid biosynthesis; and

(b) the microorganism decreases in or is deficient in the activity of the enzyme catalyzing the reaction branching from the pathway for L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid.

[0028] As examples of the enzyme catalyzing the reaction for L-glutamic acid biosynthesis of microorganisms of the genus *Enterobacter* or *Serratia*, there can be mentioned GDH, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, CS, PEPC, pyruvate dehydrogenase, pyruvate kinase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose biphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and the like. Among these enzymes, one or two or three kinds of CS, PEPC and GDH are preferred. As for the microorganism of the present invention, it is further preferred that activities of all of the three kinds of enzymes, CS, PEPC and GDH, are increased. Whether a microorganism increases in an activity of a target enzyme, and degree of the increase of the activity can be determined by measuring the enzyme activity of a bacterial cell extract or a purified fraction, and comparing it with that of a wild type strain or a parent strain.

[0029] The microorganism of the present invention, which belongs to the genus *Enterobacter* or *Serratia*, and increases in the activity of the enzyme catalyzing the reaction for L-glutamic acid biosynthesis, can be obtained as, for example, a variant where mutation has been made in a gene encoding the enzyme or a genetic recombinant strain by using any of the microorganisms mentioned above as a starting parent strain.

[0030] To enhance the activity of CS, PEPC or GDH, for example, a gene encoding CS, PEPC or GDH can be cloned in a suitable plasmid, and the aforementioned starting parent strain as a host can be transformed with the resulting plasmid. This can increase the copy number of each of the genes encoding CS, PEPC and GDH (hereinafter abbreviated as "gltA gene", "ppc gene", and "gdhA gene", respectively), and as a result the activities of CS, PEPC and GDH can be increased.

[0031] One or two or three kinds selected from the cloned gltA gene, ppc gene and gdhA gene in any combination are introduced into the starting parent strain mentioned above. When two or three kinds of the genes are introduced, either the two or three kinds of the genes are cloned in one kind of plasmid, and introduced into the host, or they are separately cloned in two or three kinds of plasmids that can exist in the same host, and introduced into the host.

[0032] The plasmid is not particularly limited so long as it can autonomously replicate in a microorganism belonging to the genus *Enterobacter* or *Serratia*. Examples of the plasmid include, for example, pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218 and the like. Other than these plasmids, phage DNA vectors can also be utilized.

[0033] Transformation can be achieved by, for example, the method of D.M. Morrison (Methods in Enzymology 68,

326 (1979)), the method by increasing permeability of recipient cells for DNA with calcium chloride (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), or the like.

[0034] The activities of CS, PEPC and GDH can also be increased by using multiple copies of the *gltA* gene, the *ppc* gene and/or the *gdh* gene present on the chromosome DNA of the starting parent strain as a host. In order to introduce multiple copies of the *gltA* gene, the *ppc* gene and/or the *gdh* gene into a chromosome DNA of a microorganism belonging to the genus *Enterobacter* or *Serratia*, sequences present on chromosome DNA in a multiple copy number such as repetitive DNA, and inverted repeats present at an end of transposition factors can be utilized. Alternatively, multiple copies of the genes can also be introduced into a chromosome DNA by utilizing transposition of transposons carrying the *gltA* gene, the *ppc* gene, or the *gdhA* gene. These techniques can increase the copy number of the *gltA* gene, the *ppc* gene, and the *gdhA* gene in transformant cells, and as a result increase the activities of CS, PEPC and GDH.

[0035] Any organisms can be used as a source of the *gltA* gene, the *ppc* gene and the *gdhA* gene used for increasing copy numbers, so long as the organisms have the CS, PEPC and GDH activities. Among such organisms, bacteria, i. e., prokaryotes, such as those bacteria belonging to the genera *Enterobacter*, *Klebsiella*, *Erwinia*, *Pantoea*, *Serratia*, *Escherichia*, *Corynebacterium*, *Brevibacterium*, and *Bacillus* are preferred. As a specific example, *Escherichia coli* can be mentioned. The *gltA* gene, the *ppc* gene and the *gdhA* gene can be obtained from a chromosome DNA of such microorganisms as mentioned above.

[0036] The *gltA* gene, the *ppc* gene and the *gdhA* gene can each be obtained from a chromosome DNA of any of the aforementioned microorganisms by isolating a DNA fragment complementing auxotrophy of a variant strain lacking the CS, PEPC or GDH activity. Alternatively, because the nucleotide sequences of these genes of bacteria of the genus *Escherichia* or *Corynebacterium* have already been elucidated (Biochemistry, Vol. 22, pp.5243-5249, 1983; J. Biochem. Vol. 95, pp.909-916, 1984; Gene, Vol. 27, pp.193-199, 1984; Microbiology, Vol. 140, pp.1817-1828, 1994; Mol. Gen. Genet. Vol. 218, pp.330-339, 1989; and Molecular Microbiology, Vol. 6, pp.317-326, 1992), the genes can be obtained by PCR using primers synthesized based on each of the elucidated nucleotide sequences, and the chromosome DNA as a template.

[0037] The activity of CS, PEPC or GDH can also be increased by, other than by the gene amplification mentioned above, enhancing expression of the *gltA* gene, the *ppc* gene or the *gdhA* gene. For example, the expression is enhanced by replacing the promoter of the *gltA* gene, the *ppc* gene, or the *gdhA* gene with another stronger promoter. Examples of such a strong promoter include, for example, a *lac* promoter, a *trp* promoter, a *trc* promoter, a *tac* promoter, a P_R promoter and a P_L promoter of lambda phage and the like. The *gltA* gene, the *ppc* gene, or the *gdhA* gene of which promoter has been substituted is cloned into a plasmid and introduced into a host microorganism, or introduced into a chromosome DNA of host microorganism using a repetitive DNA, inverted repeat, transposon or the like.

[0038] The activities of CS, PEPC or GDH can also be increased by replacing the promoter of the *gltA* gene, the *ppc* gene, or the *gdhA* gene on a chromosome with another stronger promoter (see WO87/03006, and Japanese Patent Application Laid-Open (KOKAI) No. 61-268183(1986)), or inserting a strong promoter at the upstream of each coding sequence of the genes (see Gene, 29, pp. 231-241, 1984). Specifically, these are achieved by homologous recombination between the *gltA* gene, the *ppc* gene, or the *gdhA* gene of which promoter is replaced with a stronger promoter or DNA containing a part of them, and a corresponding gene on the chromosome.

[0039] Specific examples of the microorganism belonging to the genus *Enterobacter* or *Serratia* of which CS, PEPC or GDH activity is increased include, for example, *Enterobacter agglomerans* ATCC12287/RSFCPG, *Enterobacter agglomerans* AJ13355/RSFCPG, and *Serratia liquefacience* ATCC14460/RSFCPG.

[0040] Examples of the enzyme catalyzing the reaction branching from the pathway of L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid include, for example, α KGDH, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and the like. Among these enzymes, α KGDH is preferred.

[0041] In order to obtain such decrease or deficiency of enzyme activity as mentioned above in a microorganism belonging to the genus *Enterobacter* or *Serratia*, a mutation causing the decrease or deficiency of the enzyme activity can be introduced into a gene encoding the enzyme by a conventional mutagenesis technique or genetic engineering technique.

[0042] Examples of the mutagenesis technique include, for example, the method utilizing irradiation of X-ray or ultraviolet light, the method utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine and the like. The site of gene to which a mutation is introduced may be a coding region encoding an enzyme protein, or an expression regulatory region such as a promoter.

[0043] Examples of the genetic engineering technique include, for example, genetic recombination, genetic transduction, cell fusion and the like. For example, a drug resistance gene is inserted into a target gene to produce a functionally inactivated gene (defective gene). Then, this defective gene is introduced into a cell of a microorganism belonging to the genus *Enterobacter* or *Serratia*, and the target gene on a chromosome is replaced with the defective

gene by homologous recombination (gene disruption).

[0044] Whether a microorganism decreases in an activity of a target enzyme or is deficient in the activity, and degree of the decrease of the activity can be determined by measuring the enzyme activity of a bacterial cell extract or a purified fraction of a candidate strain, and comparing it with that of a wild type strain or a parent strain. The α KGDH enzymatic activity can be measured by, for example, the method of Reed et al. (L.J. Reed and B.B. Mukherjee, Methods in Enzymology 1969, 13, p.55-61).

[0045] Depending on the target enzyme, a target variant can be selected based on a phenotype of the variant. For example, a variant which is deficient in the α KGDH activity or decreases in the activity cannot grow on a minimal medium containing glucose, or a minimal medium containing acetic acid or L-glutamic acid as an exclusive carbon source, or shows markedly reduced growth rate therein under aerobic conditions. However, even under the same condition, it can exhibit normal growth by addition of succinic acid or lysine, methionine and diaminopimelate to the minimal medium containing glucose. Based on these phenomena, a variant that is deficient in the α KGDH activity or decreases in the activity can be selected.

[0046] A method for producing a *Brevibacterium lactofermentum* strain lacking the α KGDH gene based on homologous recombination is detailed in WO95/34672, and a similar method can be used for microorganisms belonging to the genera *Enterobacter* and *Serratia*.

[0047] In addition, procedures of genetic cloning, cleavage and ligation of DNA, transformation and the like are detailed in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989) and the like.

[0048] An example of the variant strain that is deficient in the α KGDH activity or decreases in the activity obtained as described above is *Enterobacter agglomerans* AJ13356. The *Enterobacter agglomerans* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998, received an accession number of FERM P-16645, and then transferred to an international deposition under the Budapest Treaty on January 11, 1999, and received an accession number of FERM BP-6615.

[0049] The microorganism belonging to the genus *Enterobacter* or *Serratia*, and having at least one of the properties (a) and (b) and an ability to produce L-glutamic acid can be cultured in a liquid medium to produce and accumulate L-glutamic acid in the medium.

[0050] The culture medium may be an ordinary nutrient medium containing a carbon source, a nitrogen source, and inorganic salts, as well as organic trace nutrients such as amino acids, vitamins and the like, as required. It can be a synthetic medium or a natural medium. Any carbon sources and nitrogen sources can be used for the culture medium so long as they can be utilized by the microorganism to be cultured.

[0051] The carbon source may be a saccharide such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysates, molasses and the like. Further, an organic acid such as acetic acid and citric acid may also be used alone or in combination with other carbon sources.

[0052] The nitrogen source may be ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, and ammonium acetate, nitrates and the like.

[0053] As organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, materials containing them such as peptone, casamino acid, yeast extract, and soybean protein decomposition products and the like are used, and when an auxotrophic variant which requires an amino acid or the like for its growth is used, it is necessary to complement the nutrient required.

[0054] As the inorganic salt, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and the like are used.

[0055] As for the culture conditions, cultivation may be performed under aerobic conditions at a temperature of 20 to 42°C and a pH of 4 to 8. The cultivation can be continued for 10 hours to 4 days to accumulate a considerable amount of L-glutamic acid in the liquid culture medium.

[0056] After the completion of the cultivation, L-glutamic acid accumulated in the culture medium may be collected by a known method. For example, it can be isolated by a method comprising concentrating the medium after removing the cells to crystallize the product, ion exchange chromatography or the like.

Examples

[0057] The present invention will be explained more specifically with reference to the following examples.

(1) Construction of plasmid having *gluA* gene, *ppc* gene and *gdhA* gene

[0058] Procedure for construction of a plasmid having a *gluA* gene, a *ppc* gene and a *gdhA* gene will be explained by referring to Figure 1 to Figure 5.

[0059] A plasmid pBRGDH having a *gdhA* gene derived from *Escherichia coli* (Japanese Patent Application Laid-

Open (KOKAI) No. 7-203980(1995)) was digested with HindIII and *SphI*, and the both ends were blunt-ended by a treatment with T4 DNA polymerase. Then, a DNA fragment containing the *gdhA* gene was purified and collected. On the other hand, a plasmid pMWCP having a *gltA* gene and a *ppc* gene derived from *Escherichia coli* (WO97/06294) was digested with *XbaI*, and the both ends were blunt-ended by a treatment with T4 DNA polymerase. This was mixed with the DNA fragment having the *gdhA* gene purified above, and ligated with T4 ligase, giving a plasmid pMWCPG, which corresponds to the pMWCP further carrying the *gdhA* gene (Figure 1).

[0060] A DNA fragment having the *gdhA* gene obtained by digesting the pBRGDH with HindIII and *Sall* was purified and collected, and introduced into the *HindIII-Sall* site of a plasmid pSTV29 (purchased from Takara Shuzo) to obtain a plasmid pSTVG (Figure 2).

[0061] At the same time, a product obtained by digesting a plasmid pVIC40 having a replication origin of a wide-host-range plasmid RSF1010 (Japanese Patent Application Laid-Open (KOKAI) No. 8-047397(1996)) with *NotI*, followed by T4 DNA polymerase treatment and *PstI* digestion, and a product obtained by digesting pBR322 with *EcoT141*, followed by T4 DNA polymerase treatment and *PstI* digestion, were mixed and ligated with T4 ligase to obtain a plasmid RSF-Tet having the replication origin of RSF1010 and a tetracycline resistance gene (Figure 3).

[0062] Then, the pMWCPG was digested with *EcoRI* and *PstI*, and a DNA fragment having the *gltA* gene, the *ppc* gene and the *gdhA* gene was purified and collected. Similarly, the RSF-Tet was digested with *EcoRI* and *PstI*, and a DNA fragment having the replication origin of RSF1010 was purified and collected. Those DNA fragments were mixed and ligated with T4 ligase to obtain a plasmid RSFCPG composed of RSF-Tet carrying the *gltA* gene, the *ppc* gene and the *gdhA* gene (Figure 4). Expression of the *gltA* gene, the *ppc* gene and the *gdhA* gene by the resulting plasmid RSFCPG, and expression of the *gdhA* gene by the pSTVG were confirmed based on complementation of auxotrophy of *Escherichia coli* strains lacking the *gltA* gene, the *ppc* gene or the *gdhA* gene, and measurement of each enzyme activity.

[0063] A plasmid having a *gltA* gene derived from *Brevibacterium lactofermentum* was constructed as follows. PCR was performed by using primers having the nucleotide sequences represented in SEQ ID NOS: 6 and 7 selected based on the nucleotide sequence of the *gltA* gene of *Corynebacterium glutamicum* (Microbiology, 140, 1817-1828, 1994), and a chromosome DNA of *Brevibacterium lactofermentum* ATCC 13869 as a template to obtain a *gltA* gene fragment of about 3 kb. This fragment was inserted into a plasmid pHSG399 (purchased from Takara Shuzo) digested with *SmaI* to obtain a plasmid pHSGCB (Figure 5). Then, the pHSGCB was digested with HindIII, and an excised *gltA* gene fragment of about 3 kb was inserted into a plasmid pMW218 (purchased from Nippon Gene) digested with HindIII to obtain a plasmid pMWCB (Figure 5). Expression of the *gltA* gene by the resulting plasmid pMWCB was confirmed by determination of enzyme activity in the *Enterobacter agglomerans* AJ13355.

(2) Introduction of RSFCPG, pMWCB and pSTVG into *Enterobacter agglomerans* or *Serratia liquefacience*, and evaluation of L-glutamic acid productivity

[0064] The *Enterobacter agglomerans* ATCC 12287, the *Enterobacter agglomerans* AJ13355 and the *Serratia liquefacience* ATCC 14460 were transformed with the RSFCPG, pMWCB and pSTVG by electroporation (Miller J.H., "A Short Course in Bacterial Genetics; Handbook" Cold Spring Harbor Laboratory Press, USA, 1992) to obtain transformants exhibiting tetracycline resistance.

[0065] Each of the resulting transformants and the parent strains was inoculated into 50 ml-volume large size test tube containing 5 ml of a culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, and 30 g/L calcium carbonate, and cultured at 37°C with shaking until the glucose contained in the culture medium was consumed. However, as for the AJ13355/pMWCB strain and the AJ13355/pSTVG strain, the cultivation was stopped when about 10 g/L of glucose was consumed, i.e., cultivated for 15 hours like the parent strain AJ13355, because their glucose consumption rates were low. To the culture medium of the transformants, 25 mg/L of tetracycline was added. After the cultivation was completed, L-glutamic acid accumulated in the culture medium was measured. The results are shown in Table 1.

Table 1:

Accumulated amount of L-glutamic acid	
Bacterial strain	Accumulated amount of L-glutamic acid
ATCC12287	0.0 g/L
ATCC12287/RSFCPG	6.1

Table 1: (continued)

Accumulated amount of L-glutamic acid	
Bacterial strain	Accumulated amount of L-glutamic acid
AJ13355	0.0
AJ13355/RSFCPG	3.3
AJ13355/pMWCB	0.8
AJ13355/pSTVG	0.8
ATCC14460	0.0
ATCC14460/RSFCPG	2.8
Culture medium alone	0.2

[0066] While the *Enterobacter agglomerans* ATCC12287, the *Enterobacter agglomerans* AJ13355 and the *Serratia liquefaciens* ATCC14460 did not accumulate L-glutamic acid, the strains whose CS, PEPC and GDH activities were amplified by introducing RSFCPG accumulated 6.1 g/L, 3.3 g/L, and 2.8 g/L of L-glutamic acid, respectively. The AJ13355 strain of which CS activity alone was amplified accumulated 0.8 g/L of L-glutamic acid, and the strain of which GDH activity alone was amplified also accumulated 0.8 g/L of L-glutamic acid.

(3) Cloning of α KGDH gene (referred to as "sucAB" hereinafter) of *Enterobacter agglomerans* AJ13355

[0067] The *sucAB* gene of the *Enterobacter agglomerans* AJ13355 was cloned by selecting a DNA fragment complementing acetate non-assimilation of an *Escherichia coli* strain lacking the α KGDH-E1 subunit gene (referred to as "sucA" hereinafter) from the chromosome DNA of the *Enterobacter agglomerans* AJ13355.

[0068] The chromosome DNA of the *Enterobacter agglomerans* AJ13355 strain was isolated by the same method as conventionally used for extracting chromosome DNA from *Escherichia coli* (Seibutsu Kogaku Jikken-sho (Textbook of Bioengineering Experiments), Ed. by the Society of Fermentation and Bioengineering, Japan, p.97-98, Baifukan, 1992). The pTWV228 used as the vector (ampicillin resistant) was a marketed product from Takara Shuzo.

[0069] Products obtained by digesting the chromosome DNA of the AJ13355 strain with *Eco*T221 and products obtained by digesting the pTWV228 with *Pst*I were ligated by T4 ligase, and the *Escherichia coli* JRG465 lacking *sucA* (Herbert J. et al., Mol. Gen. Genetics, 1969, 105, p.182) was transformed with them. Strains grown on the acetic acid minimal medium were selected from the transformants obtained as described above, and a plasmid extracted from them was designated as pTWVEK101. The *Escherichia coli* JRG465 carrying the pTWVEK101 recovered the characteristics of acetate non-assimilability as well as auxotrophy for succinic acid or L-lysine and L-methionine. This suggests that the pTWVEK101 contains the *sucA* gene of *Enterobacter agglomerans*.

[0070] A restriction map of *Enterobacter agglomerans*-derived DNA fragment of pTWVEK101 is shown in Figure 6. The result of nucleotide sequencing of the hatched portion in Figure 6 is shown in SEQ ID NO: 1. In this sequence, two full length ORFs and two nucleotide sequences considered as partial sequences of ORFs were found. Amino acid sequences that can be encoded by these ORFs and the partial sequences thereof are shown in SEQ ID NOS: 2 to 5 in order from the 5' ends. As a result of homology analysis of these sequences, it was found that the portion of which nucleotide sequence had been determined contained a 3' partial sequence of succinate dehydrogenase iron-sulfur protein gene (*sdhB*), full length *sucA* and α KGDH-E2 subunit gene (*sucB* gene), and 5' partial sequence of succinyl-CoA synthetase β subunit gene (*sucC* gene). Comparison of the amino acid sequences deduced from these nucleotide sequences with those of *Escherichia coli*. (Eur. J. Biochem., 141, 351-359 (1984), Eur. J. Biochem., 141, 361-374 (1984), and Biochemistry, 24, 6245-6252 (1985)) is shown in Figures 7 to 9. As shown by these results, the amino acid sequences exhibited markedly high homology. It was also found that a cluster of *sdhB-sucA-sucB-sucC* is formed on the *Enterobacter agglomerans* chromosome like *Escherichia coli* (Eur. J. Biochem., 141, 351-359 (1984), Eur. J. Biochem., 141, 361-374 (1984), and Biochemistry, 24, 6245-6252 (1985)).

(4) Acquisition of strain deficient in α KGDH derived from *Enterobacter agglomerans* AJ13355

[0071] Using the *sucAB* gene of *Enterobacter agglomerans* obtained as described above, a strain lacking α KGDH of *Enterobacter agglomerans* was obtained by homologous recombination.

[0072] First, pTWVEK101 was digested with *Bgl*III to remove the C-terminus region corresponding to about half of the *sucA* gene and the full length of the *sucB* gene. To this site, a chloramphenicol resistance gene fragment cut out from the pHS399 (Takara Shuzo) with *Acc*I was then inserted. The region of *sdhB- Δ sucAB::Cm^r-sucC* obtained above was cut out with *A*III and *Sac*I. The resulting DNA fragment was used to transform the *Enterobacter agglomerans*

AJ13355 strain by electroporation to obtain a chloramphenicol resistant strain, and thus a *Enterobacter agglomerans* AJ13356 strain lacking the *sucAB* gene where the *sucAB* gene on the chromosome was replaced by *sucAB::Cm^r* was obtained.

[0073] To confirm that the AJ13356 strain obtained as described above was deficient in the α KGDH activity, its enzymatic activity was determined by the method of Reed (L.J. Reed and B.B. Mukherjee, Methods in Enzymology 1969, 13, p.55-61). As a result, the α KGDH activity could not be detected in the AJ13356 strain as shown in Table 2, and thus it was confirmed that the strain lacked the *sucAB* as desired.

Table 2:

α KGDH activity	
Bacterial strain	α KGDH activity (Δ ABS/min/mg protein)
AJ13355	0.481
AJ13356	<0.0001

(5) Evaluation of L-glutamic acid productivity of *Enterobacter agglomerans* strain deficient in α KGDH

[0074] Each of the AJ13355 and AJ13356 strains was inoculated into a 500 ml-volume flask containing 20 ml of a culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, 30 g/L calcium carbonate, 200 mg/L L-lysine monohydrochloride, 200 mg/L L-methionine and 200 mg/L DL- α,ϵ -diaminopimelic acid (DAP), and cultured at 37°C with shaking until the glucose contained in the culture medium was consumed. After the cultivation was completed, L-glutamic acid and α -ketoglutaric acid (abbreviated as " α KG" hereinafter) accumulated in the culture medium were measured. The results are shown in Table 3.

Table 3:

Accumulated amounts of L-glutamic acid and α KG		
Bacterial strain	Accumulated amount of L-glutamic acid	Accumulated amount of α KG
AJ13355	0.0 g/L	0.0 g/L
AJ13356	1.5	3.2

[0075] The AJ13356 strain deficient in the α KGDH activity accumulated 1.5 g/L of L-glutamic acid, and simultaneously accumulated 3.2 g/L of α KG.

(6) Introduction of RSFCPG into *Enterobacter agglomerans* strain lacking α KGDH and evaluation of L-glutamic acid productivity

[0076] The AJ13356 strain was transformed with the RSFCPG, and the resulting strain introduced with the RSFCPG, AJ13356/RSFCPG, was inoculated into a 500 ml-volume flask containing 20 ml of a culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, 25 mg/L tetracycline, 30 g/L calcium carbonate, 200 mg/L L-lysine monohydrochloride, 200 mg/L L-methionine and 200 mg/L DL- α,ϵ -DAP, and cultured at 37°C with shaking until the glucose contained in the culture medium was consumed. After the cultivation was completed, L-glutamic acid and α KG accumulated in the culture medium were measured. The results are shown in Table 4.

Table 4:

Accumulated amounts of L-glutamic acid and α KG		
Bacterial strain	Accumulated amount of L-glutamic acid	Accumulated amount of α KG
AJ13356	1.4 g/L	2.9 g/L

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Table 4: (continued)

Accumulated amounts of L-glutamic acid and α KG		
Bacterial strain	Accumulated amount of L-glutamic acid	Accumulated amount of α KG
AJ13356/RSFCPG	5.1	0.0

[0077] In the strain of which CS, PEPC and GDH activities were amplified by the introduction of RSFCPG, the accumulated amount of α KG was reduced, and the accumulated amount of L-glutamic acid was further improved.

Annex to th description

[0078]

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SEQUENCE LISTING

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	Asp Ala Leu Asn Val Asn Met Ser Ser Val Asp	
	930 935 1 5	
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	Ile Leu Val Pro Asp Leu Pro Glu Ser Val Ala Asp Ala Thr Val Ala	
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	Thr Trp His Lys Lys Pro Gly Asp Ala Val Ser Arg Asp Glu Val Ile	
	25 30 35	
	gtc gaa att gaa act gac aaa gtc gtg ctg gaa gtg ccg gca tct gcc	3303
	Val Glu Ile Glu Thr Asp Lys Val Val Leu Glu Val Pro Ala Ser Ala	
50	40 45 50	
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	Asp Gly Val Leu Glu Ala Val Leu Glu Asp Glu Gly Ala Thr Val Thr	
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	Glu Ser Ser Ala Lys Ala Glu Ser Asn Asp Thr Thr Pro Ala Gln Arg							
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	Tyr	Phe	Arg	Arg	Leu	Ala	Lys
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10	Thr	Asp	Pro	Ala	Thr	Asn	Ser
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	Asn	Ala	Phe	Arg	Phe	Arg	Gly
				100		105	
15	Gly	Leu	Trp	Lys	Gln	Asp	Arg
				115		120	
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20	Ala	Ile	Gly	Lys	Glu	Thr	Met
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	Ala	Lys	Ile	Tyr	Asn	Asp	Arg	Lys	Leu	Met	Ala	Glu	Gly	Glu	Lys	Ala
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	Tyr	Thr	Pro	Leu	His	His	Ile	His	Asn	Ser	Gln	Gly	Glu	Phe	Lys	Val
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	Tyr	Ala	Thr	Ala	Glu	Pro	Arg	Val	Leu	Thr	Ile	Trp	Glu	Ala	Gln	Phe
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 Ser Ile Asp Gly Glu Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser
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30

Claims

1. A microorganism belonging to the genus *Enterobacter* or *Serratia* having the ability to produce L-glutamic acid and having at least one of the following properties:

- (a) the microorganism has increased activity of an enzyme catalyzing a reaction in the L-glutamic acid biosynthesis; and
- (b) the microorganism has decreased activity or is deficient in an activity of an enzyme catalyzing a reaction branching from the pathway of the L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid.

2. A microorganism according to claim 1 wherein the enzyme catalyzing the reaction in the L-glutamic acid biosynthesis is at least one selected from the group consisting of citrate synthase, phosphoenolpyruvate carboxylase, and glutamate dehydrogenase.

3. A microorganism according to claim 2 wherein the enzyme catalyzing the reaction in the L-glutamic acid biosynthesis includes all of citrate synthase, phosphoenolpyruvate carboxylase, and glutamate dehydrogenase.

4. A microorganism according to any one of claims 1 to 3 wherein the enzyme catalyzing the reaction branching from the pathway of the L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid is α -ketoglutarate dehydrogenase.

5. A microorganism according to any one of claims 1 to 4 which is *Enterobacter agglomerans* or *Serratia liquefaciens*.

6. A method for producing L-glutamic acid which comprises culturing the microorganism as defined in any one of claims 1 to 5 in a liquid culture medium to produce and accumulate L-glutamic acid in the culture medium, and collecting the L-glutamic acid from the culture medium.

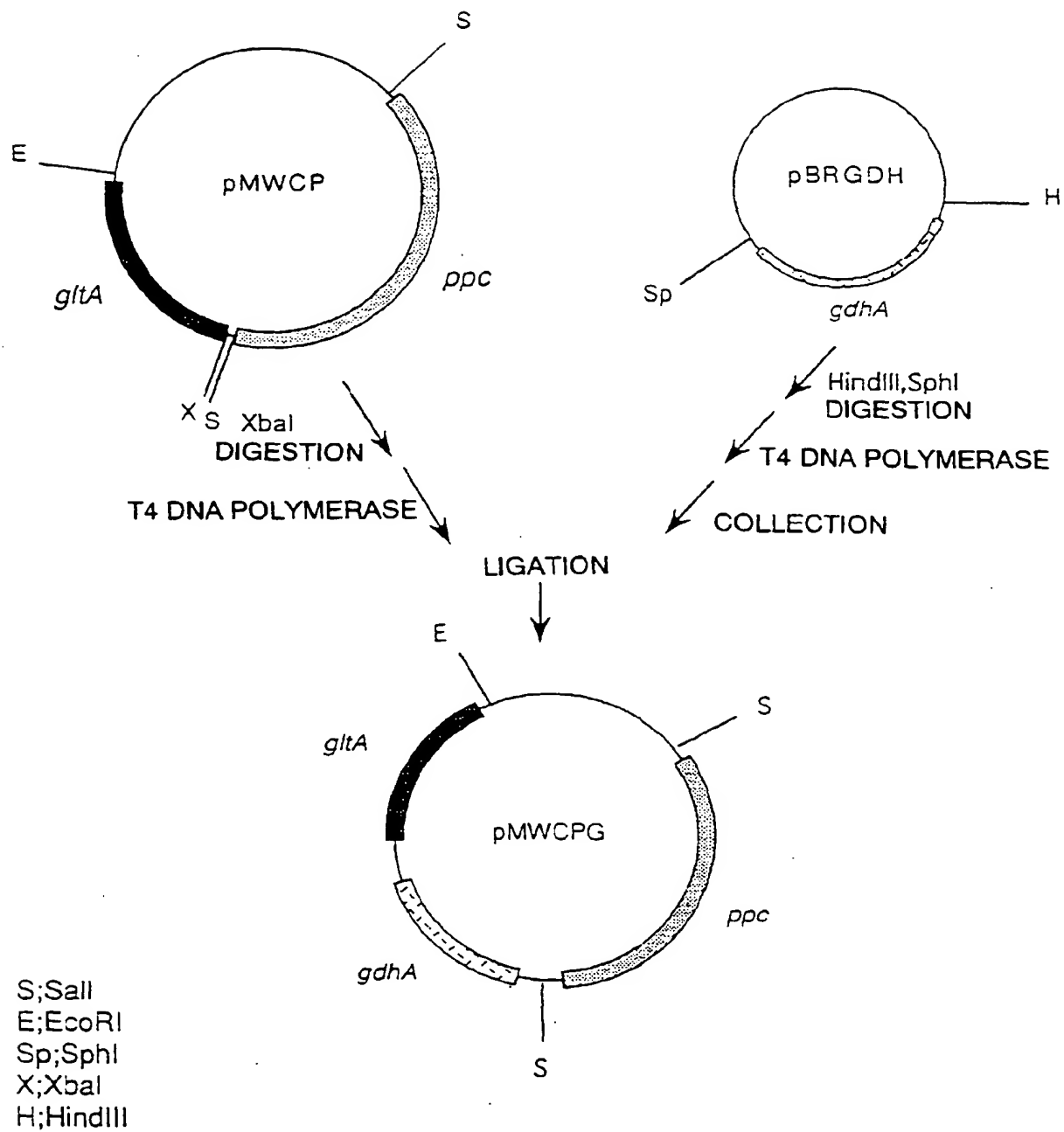


FIG. 1

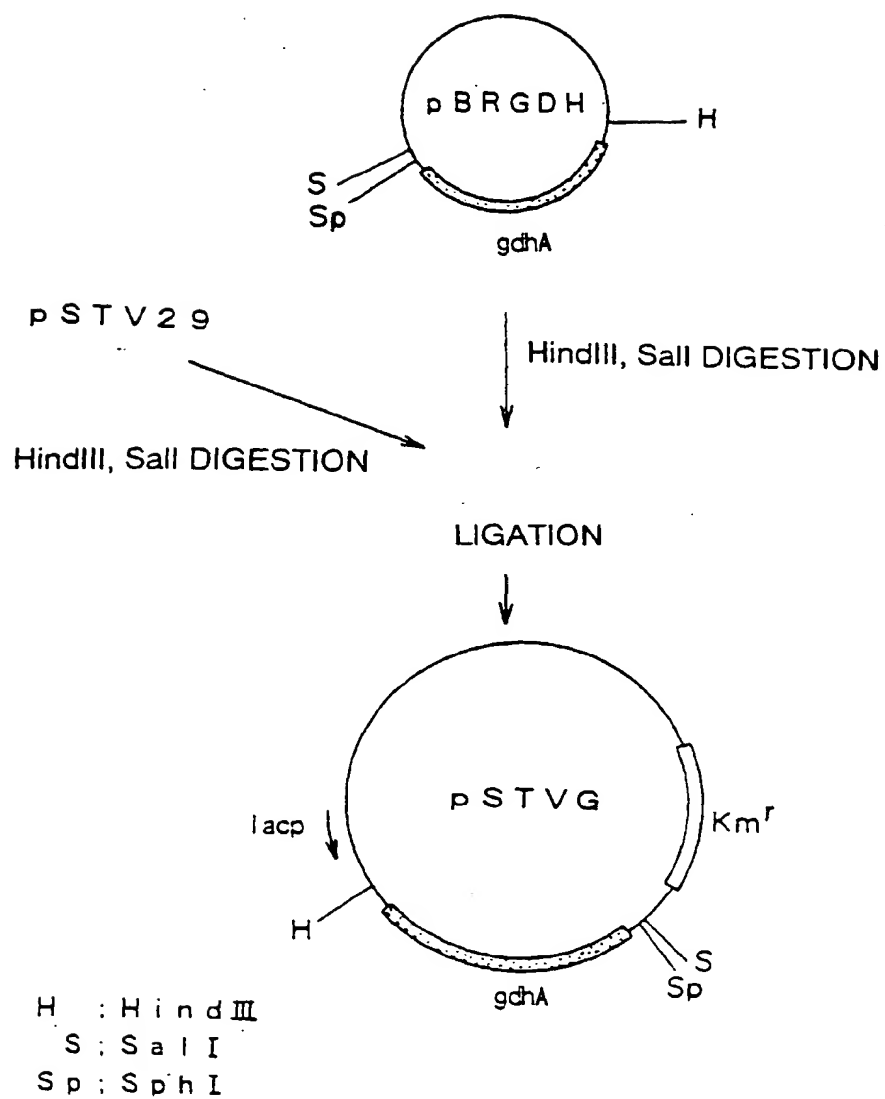


FIG. 2

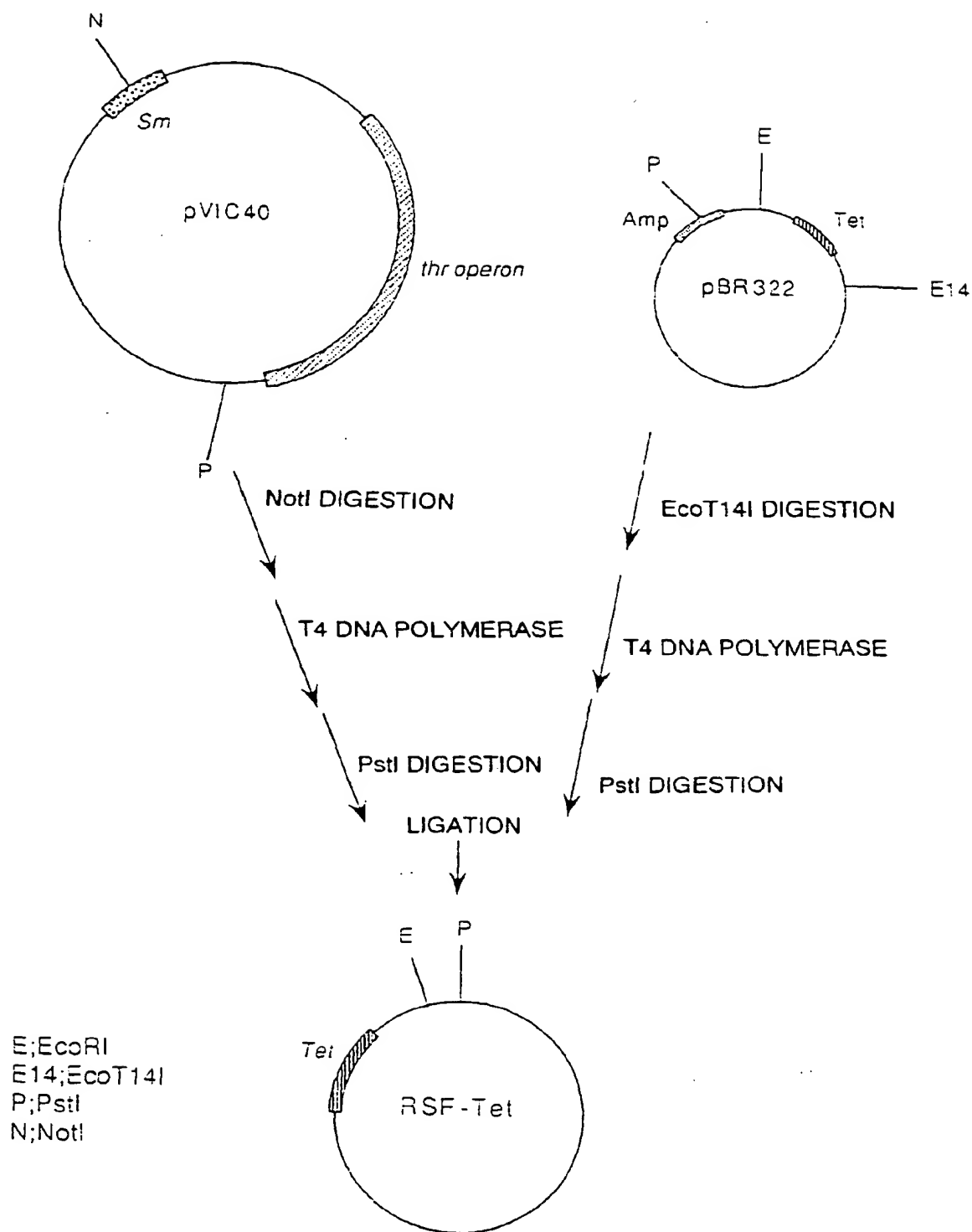


FIG. 3

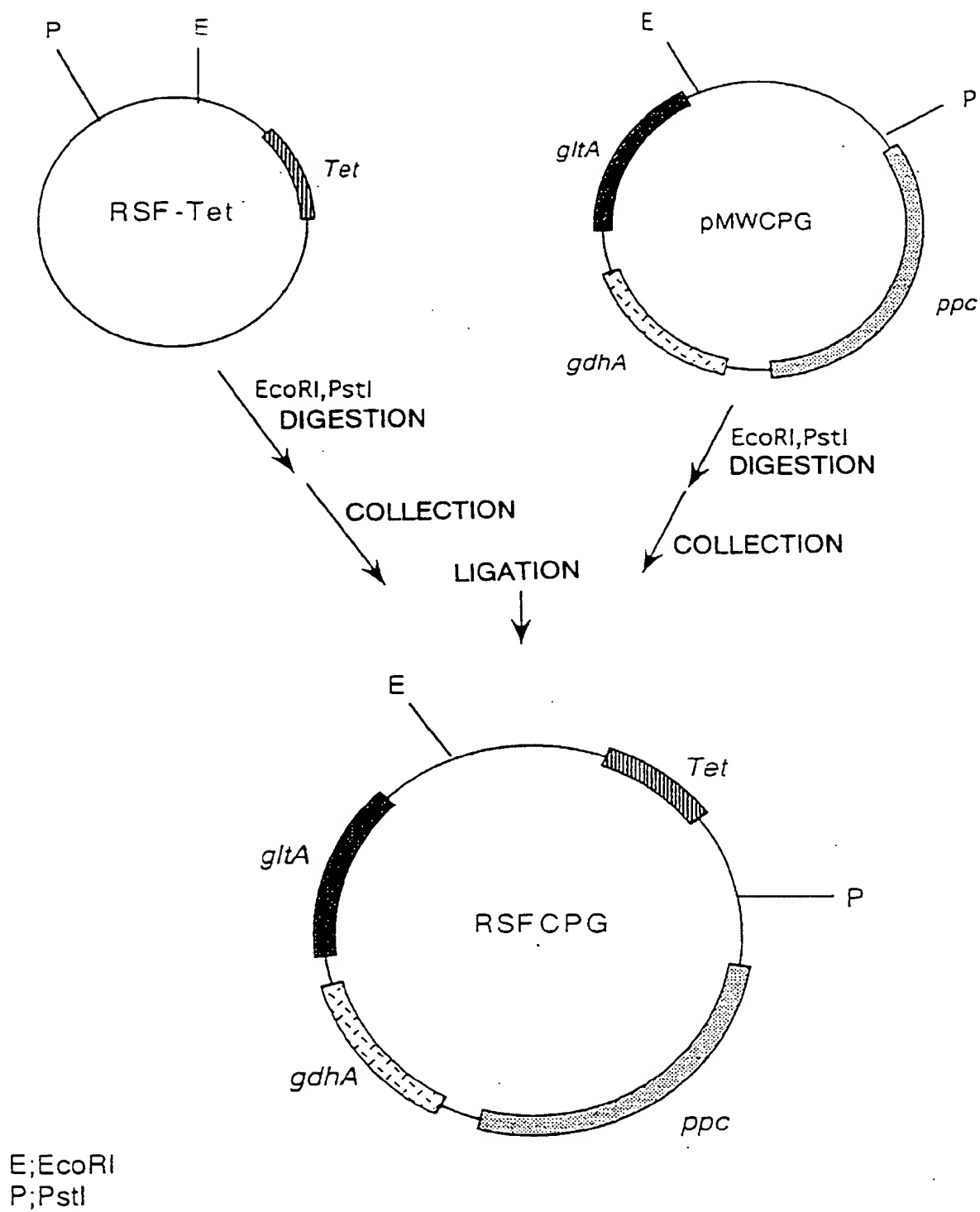


FIG. 4

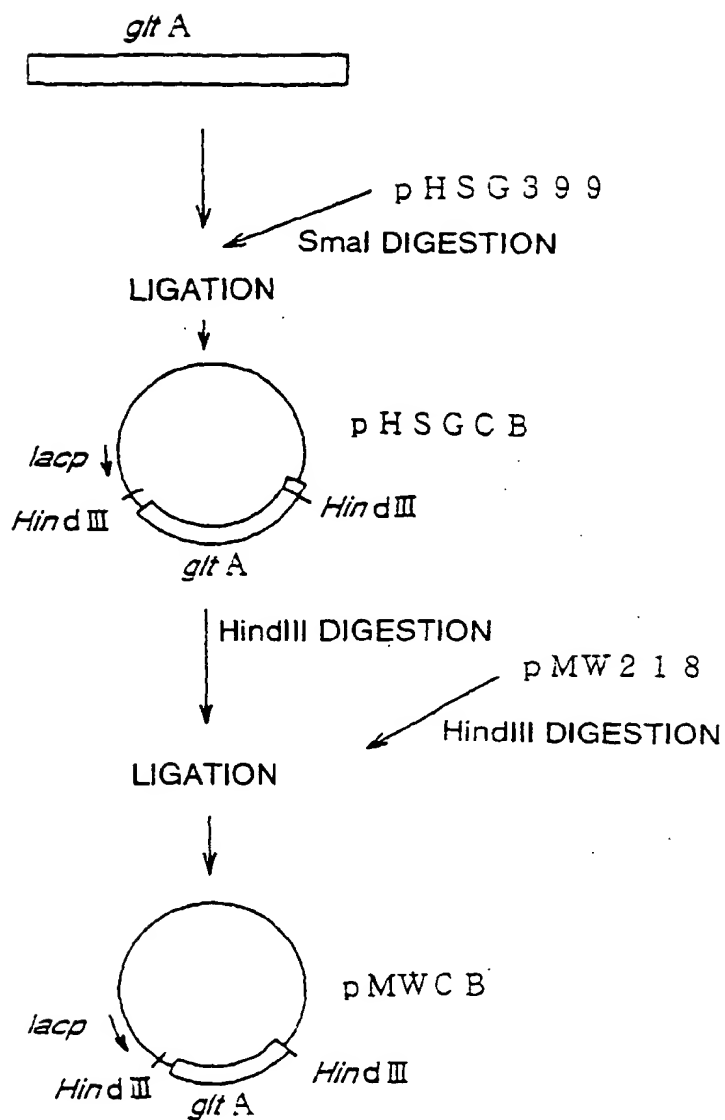


FIG. 5

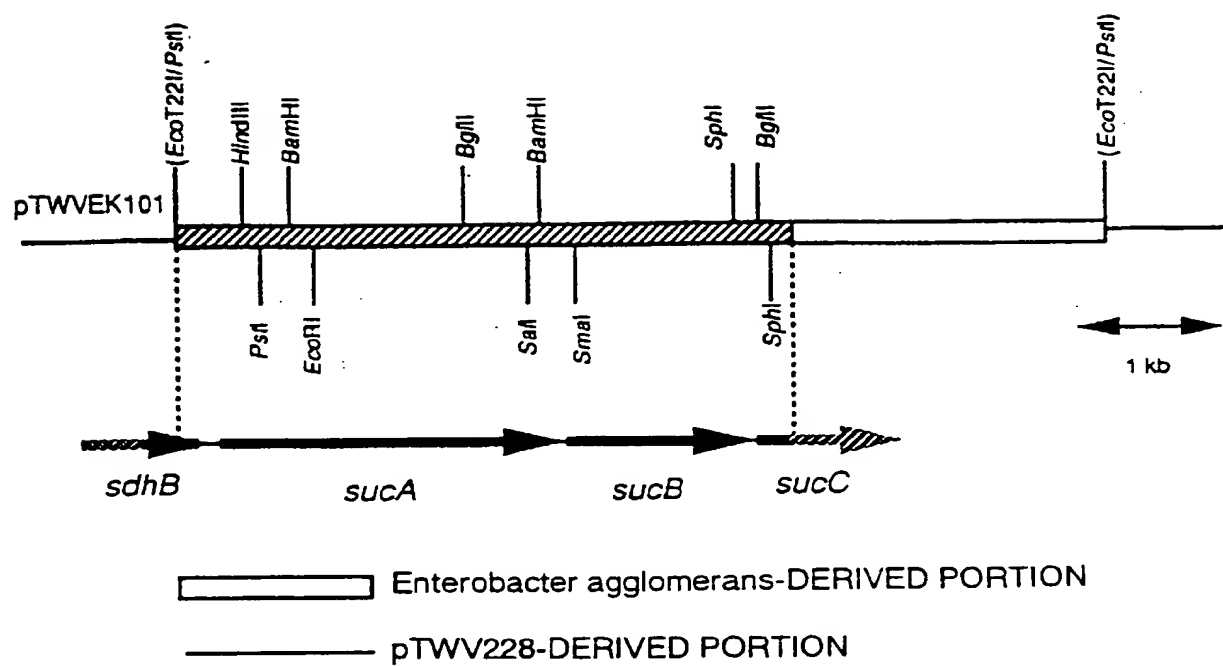


FIG. 6

[88.0% / 935 aa]

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539' HEWDEEYPNKVEMKRLQELAKRISTVPEAVEMQSRVAKIYGDRQAMAAGEKLFDWGGAEN
601' LAYATLVDEGIPVRLSGEDSGRGTFFHRHAVHNQANGSTYTPLHHI HNSQGEFKVWDSV
599' LAYATLVDEGIPVRLSGEDSGRGTFFHRHAVIHNQNGSTYTPLQHI HNGQGAFRVWDSV
661' LSEEAVLAFEYGYATAEPRLT IWEAQFGDFANGAQVVIDQFISSGEQKNGRMCGLVM LL
659' LSEEAVLAFEYGYATAEPRTL IWEAQFGDFANGAQVVIDQFISSGEQKNGRMCGLVM LL
721' PHGYEGQGPEHSSARLERYLQLCAEQNMQVCVPSTPAQVYHMLRRQALRGMRRLVVMSP
719' PHGYEGQGPEHSSARLERYLQLCAEQNMQVCVPSTPAQVYHMLRRQALRGMRRLVVMSP
781' KSLLRHPLAIISSLELANGSFQPAIGEIDDLDPQGKRVVLC SGKVYYDLLEQRRKDEKT
779' KSLLRHPLAVSSLEELANGTFLPAIGEIDELDPKGKRVVMCSGKVYYDLLEQRRKNNQH
841' DVAIVRIEQLYPFPHQAVQEALKAYSHVQDFVMCQEEPLNQGAWYCSQH HFRDVPFGAT
839' DVAIVRIEQLYPFPHKAMQEVLLQFAHYKDFVMCQEEPLNQGAWYCSQH HFREVIPFGAS
901' LRYAGRPASASPAVGYSVHQQQQDLVNDALNVN
899' LRYAGRPASASPAVGYSVHQQQQDLVNDALNVE

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FIG. 7

[88.2% / 407 aa]

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1' MSSVDILVPDLPESVADATVATWHKKPGDAVSRDEIVEIETDKVVLVPASADGVLEAV
.....
1' MSSVDILVPDLPESVADATVATWHKKPGDAVVRDEVLEIETDKVVLVPASADGILDAV
.....
61' LEDEGATVTSRQILGRLKEGNSAGKESSAKAESNDTPAQRQTASLEEESDALSPAIRR
.....
61' LEDEGTTVTSRQILGRLREGNSAGKETSAKSEEEKASTPAQRQQASLEEQNNDALSPAIRR
.....
121' LIAEHNLDAAQIKGTGVGGRLTREDVEKHLANKPQAEKAAAPAAGAATAQQPVANRSEKR
.....
121' LLAEHNLDASAIKGTGVGGRLTREDVEKHLAKAPAKE--SAPAAAAAPAAQPALAARSEKR
.....
181' VPMTRLRKRVAERLLEAKNSTAMLTTFNEINMKPIMDLRKQYGDAFEKRGVRLGFMSFY
.....
179' VPMTRLRKRVAERLLEAKNSTAMLTTFNEVNMKPIMDLRKQYGEAFEKRGIRLGFMSFY
.....
241' IKAVVEALKRYPEVNASIDGEDVYHNYFDVSIADVSTPRGLVTPVLRDVALSMADIEKK
.....
239' VKAVVEALKRYPEVNASIDGDDVYHNYFDVSMADVSTPRGLVTPVLRDVALSMADIEKK
.....
301' IKELAVKGRDGKLTVDLTGGNFTITNGGVFGSLMSTPIINPPQSAILGMHAIKDRPMAY
.....
299' IKELAVKGRDGKLTVEDLTGGNFTITNGGVFGSLMSTPIINPPQSAILGMHAIKDRPMAY
.....
361' NGQVVILPMMYLALSYDHRLIDGRESVGYLVAVKEMLEDPARLLLDV
.....
359' NGQVEILPMMYLALSYDHRLIDGRESVGYLVAVKEMLEDPTRLLLDV

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FIG. 8

[95.1% / 41 aa]

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1' MNLHEYQAKQLFARYGMPAPTGYACTTPREAEAAASKIGAG
.....
1' MNLHEYQAKQLFARYGLPAPVGYACTTPREAEAAASKIGAGPMVVKCQVHAGGRGKAGGV

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FIG. 9

[97.4% / 39 aa]

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1' AFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMMLLQRSA
.....
181' FLIDSRDTETDSRLDGLSDAFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMMLLQRNA

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FIG. 10